Insect Biochem Vol 15 No 4 pp 521–528, 1985
Printed in Great Britain

0020-1790/85 $3 00 + 0 00
Pergamon Press Ltd

STABILIZATION OF MINERALIZED AND SCLEROTIZED PUPARIAL CUTICLE OF MUSCID FLIES

CRAIG R ROSELAND*, MICHAEL J GRODOWITZ*, KARL J KRAMER†‡§, THEODORE L HOPKINS* and ALBERTO B BROCE*

Departments of Entomology* and Biochemistry†, Kansas State University, Manhattan, KS 66506 and ‡U S Grain Marketing Research Laboratory Agricultural Research Service, U S Department of Agriculture, Manhattan, KS 66502, U S A

(*Received* 3 *June* 1984, *revised* 24 *August* 1984)

Abstract—Calcium, magnesium and phosphorus are the major mineral elements in puparial exuviae of the face fly, *Musca autumnalis* house fly, *M domestica* and stable fly, *Stomoxys calcitrans*, but they are 20–50 times more prevalent in face fly tha in the other two species that sclerotize the puparium Carbon and nitrogen are approx 5 times more abundant in house fly puparia than in face fly puparia Face fly puparia contain two and three-fold less total amino acids than the house fly and stable fly, respectively β-Alanine is a major amino acid in puparial cuticle of the house fly and stable fly, but it is absent in the face fly There is no significant difference in glucosamine (chitin) content between the three species Dopamine is the major catechol detected in face fly puparial cuticle while *N*-β-alanyldopamine (NBAD) is 10 to 15 times more prevalent than other catechols such as dopamine, *N*-acetyldopamine (NADA), 3,4-dihydroxyphenylalanine (DOPA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in house fly and stable fly puparial cuticles The latter two species have 75 to nearly 200 times higher levels of extractable catechols than the face fly At the onset of pupariation, dopamine and NBAD attain nearly equivalent titres in puparial cuticles of face fly and house fly, respectively Dopamine subsequently decreases more than 40-fold in the face fly as the cuticle becomes stabilized, while NBAD continues to accumulate in the house fly The house fly covalently incorporates about 150 times more catechols in the puparium than does the face fly The force required to fracture house fly and stable fly puparia is about three-fold greater than that required to fracture face fly puparia of comparable thickness However, the face fly puparium attains a strength comparable to those of house fly and stable fly puparia by significantly increasing its thickness These results demonstrate that dipterans use both catecholamines and minerals for stabilization of puparial cuticle with the house fly and stable fly relying primarily on sclerotization and the face fly on mineralization

Key Word Index Catecholamines, minerals, sclerotization, pupariation, Diptera, tanning, dopamine, DOPA, *N*-β-alanyldopamine, cuticle, *N*-acetyldopamine, tyrosine metabolism, β-alanine, calcium, magnesium, phosphorus, face fly, house fly, stable fly, cuticular filler protein, mineralization, calcification, glucosamine, chitin

INTRODUCTION

Insect cuticle is hardened and stabilized when tyrosine derivatives are incorporated into the most external layers of the chitin-protein matrix Quinonoid metabolites apparently crosslink proteins and are believed to be the principal means of cuticle sclerotization (reviewed by Neville, 1975, Brunet, 1980, Lipke *et al*, 1983) However, a dehydrating mechanism involving catechols that impregnate cuticle may also contribute to its stabilization (Fraenkel and Rudall, 1940, Vincent and Hillerton, 1979) For example, as sclerotization proceeds, catecholamines extractable in acid have been shown to progressively accumulate in various cuticles (Hopkins *et al*, 1982, 1984)

Typically Diptera metabolize tyrosine to *N*-acetyldopamine for the purpose of puparial tanning (Karlson and Sekeris, 1962, Sekeris and Herrlich, 1966) The face fly, *Musca autumnalis* (De Geer), however, appears to be an exception to this mechanism of puparial hardening Fraenkel and Hsiao (1967) reported that the deposition of calcium salts account for hardening and stabilization of the puparium in this species Evidence to support their hypothesis that calcification supplants sclerotization in face fly puparia included (1) the presence of high levels of calcium salts, (2) limited decreases in extractable protein in cuticle during puparial hardening and (3) no decline in tyrosine titre in whole animals during pupariation In contrast, large decreases in tyrosine content and extractable cuticular protein occurred in the house fly, *Musca domestica* (L), during pupariation Darlington *et al* (1983) reexamined the mineral and organic composition of face fly puparia and observed that, although mineral salts accounted for the bulk of the material in the puparial cuticle, sclerotization could not be ruled out since minor amounts of water soluble protein were present after hardening Another significant difference between face fly and house fly puparial cuticles is the absence of β-alanine (3-amino propionic acid) in the face fly (Bodnaryk, 1972) β-Alanine is present in large quantity in house fly puparia and in puparia of other Diptera that normally form dark brown sclerotized cuticle (Dennell, 1958, Fukushi and Seki, 1965,

§Send correspondence to K J Kramer, U S Grain Marketing Research Laboratory, 1515 College Avenue, Manhattan KS 66502 U S A

Hackman and Goldberg, 1971 Bodnaryk, 1972) β-Alanine is a constituent of the dopamine metabolite, *N*-β-alanyldopamine (NBAD) which has been found in many insect species including Diptera (Hopkins *et al*, 1982 1984, Kramer *et al* 1984) The lack of β-alanine in face fly cuticle therefore also suggests that sclerotization is not the primary mechanism for puparial hardening but does not entirely rule out a role for tyrosine metabolites in the stabilization process

To test more directly the possibility that catecholamines are involved in stabilization of puparial cuticle, we have determined their titres in larval and puparial cuticles of the face fly For comparison similar studies have been conducted with the closely related house fly and the stable fly, *Stomoxys calcitrans* (L) These species form a typical dark brown sclerotized puparium in contrast to the white puparium of the face fly Mineral and amino acid compositions of the puparia were also measured to further examine the similarities and differences in the mechanism of stabilization and hardening of the puparial cuticle of three species of Diptera In addition, we have determined the force required to fracture puparial exuviae in order to quantitate the relative hardness of mineralized and sclerotized puparial cuticles

MATERIALS AND METHODS

Insects

Face flies house flies and stable flies were obtained from colonies maintained at the Department of Entomology Kansas State University Face flies were reared on fresh bovine manure house flies on CSMA medium (Chemical Specialities Manufacturers Association Ralston-Purina St Louis Missouri) and stable flies on a modified corn cob-fish meal medium Wandering larvae of the face fly were identified as those individuals which left the medium after four or five days at 25 C while wandering larvae of the house fly and stable fly were those which had completely emptied their digestive tracts Occasionally larvae were maintained at 18 C to slow development The onset of puparium formation is described as the time that larval cuticle had contracted into the typical puparial shape (anterior retraction) and larval movement had ceased

Determination of catechols

Larvae and puparia of appropriate age were chilled on ice and dissected in cold Drosophilan saline solution Epidermis and muscles were scraped from the cuticle The cleaned cuticle was homogenized in 1 2 M HCl and 0 4 mM sodium metabisulfite in ground glass tissue grinders Catechols were recovered after adsorption to alumina and analyzed by reverse phase liquid chromatography with electrochemical detection (Hopkins *et al* 1982 1984) The mobile phase was 17 8% methanol 0 6% phosphoric acid and 98–146 mg sodium octyl sulfate per liter at pH 3 05 Retention times of cuticular catechols were compared to those of standard compounds To confirm the identification of some of the metabolites in homogenates a second chromatographic separation was performed that utilized a mobile phase consisting of acetonitrile (25%) and sodium lauryl sulfate (316 mg per liter) instead of methanol and sodium octyl sulfate (T D Morgan personal communication)

Amino acid analysis

Aliquots of cuticle homogenates were mixed with equal volumes of concentrated HCl Hydrolysis was carried out for 20 hr at 110 C *in vacuo* Amino acids and glucosamine were separated by cation exchange chromatography and detected by post column ninhydrin derivatization

Elemental analysis

Puparial exuviae were washed in deionized water scraped free of adhering internal membranes and meconia ashed and analyzed by multichannel graphite furnace atomic absorption spectroscopy at the Kansas State University Emission Spectroscopy Laboratory The ash weights represent single determinations Carbon hydrogen oxygen nitrogen and sulfur were determined by Huffman Laboratories Inc (Wheatridge Colorado)

Incorporation of DOPA into cuticle

Wandering stage larvae were injected with [2-^{14}C]DOPA (Roseland *et al* 1983) and then puparia were collected after adult eclosion The puparial cuticle was prepared and catecholamines were extracted and analyzed as previously described The unextracted residues were combusted in a Packard Tri-Carb Sample Oxidizer for collection of ^{14}CO Distribution of radioactivity was determined by liquid scintillation counting

Measurement of puparial thickness diameter hardness and density

Cuticular thickness was measured using an ETEC Auto Scan U-1 scanning electron microscope Puparial diameter was determined with an ocular micrometer in a stereomicroscope The relative hardness of puparial exuviae was measured with an Instron® Tensile Tester Model 1132 with a 2 kg load cell equipped with a probe modified for small pieces of cuticle The probe diameter was 0 61 mm and the base plate aperture through which the probe entered was 0 71 mm The density of puparial exuviae of face fly and house fly was determined by calculations assuming that the exuviae were of a uniform cylindrical shape The posterior end of the exuviae was removed and the diameter and length determined under the stereomicroscope Thickness was measured using a compound microscope with epi-illumination Weight was obtained using a Cahn electrobalance

RESULTS

Elemental composition

We initially compared the mineral compositions of puparial cuticle from the three species of flies by measuring the ash weight and some of the inorganic constituents of the exuviae (Table 1) As was observed by Darlington *et al* (1983) we found the percentage of puparial weight as ash in face fly exuviae to be about 63% approximately four times greater than the ash content in sclerotized puparia of the house fly and stable fly Calcium phosphorus and magnesium were the predominant inorganic elements in that relative order of magnitude Sclerotized puparial exuviae of the house fly and stable fly contained from 20 to over 40 times less calcium than face fly on a percentage weight basis Phosphorus and magnesium were also at much reduced levels in those species The face fly cuticle exhibited only moderately greater levels of potassium, zinc and manganese than either house fly or stable fly cuticle and there were similar low levels of copper iron and cadmium in all species The house fly consistently had larger percentages of the major inorganic elements than the stable fly Our results confirm that calcium phosphorus and magnesium are far more prevalent in face fly puparial exuviae (31 4% of dry weight) than in the other fly species The house fly and stable fly also deposit lower amounts of minerals (2–4% of dry weight) primarily in the form of calcium and phos-

Table 1 Ash and major inorganic elements as percentage of dry weight of puparial exuviae of three species of Diptera*

Species	Ash	Elements (weight %)									
		Ca	P	Mg	K	Zn	Mn	Cu	Fe	Cd	Co
Musca autumnalis (face fly)	62 77	18 47 ± 1 01	9 93 ± 0 20	3 03 ± 0 03	0 89 ± 0 04	0 24 ± 0 07	0 15 ± 0 09	0 06 ± 0 01	0 06 ± 0 01	0 03 ± 0 01	< 0 06
Musca domestica (house fly)	3 65	0 95 ± 0 05	0 31 ± 0 01	0 29 ± 0 02	0 22 ± 0 02	0 16 ± 0 01	0 08 ± 0 01	0 07 ± 0 01	0 05 ± 0 01	0 04 ± 0 01	< 0 06
Stomoxys calcitrans (stable fly)	2 31	0 40 ± 0 60	0 20 ± 0 03	0 04 ± 0 01	0 05 ± 0 04	0 12 ± 0 01	< 0 01	0 05 ± 0 01	0 06 ± 0 01	0 04 ± 0 01	< 0 06
Detection limits		0 01	0 11	0 02	0 02	0 03	0 01	0 01	0 01	0 01	0 06

*Mean of three determinations ± SE

phorus (also magnesium and potassium in the house fly) in their puparial cuticles Apparently, puparial mineralization involves a significant deposition of magnesium salt as well as that of calcium Darlington *et al* (1983) determined that calcium and magnesium phosphates are the major salts in empty puparia of the face fly with lesser contributions from calcium and magnesium carbonates Similar elemental compositions have been reported for puparia of *Musca fergusoni*, a closely related species which uses minerals for puparial stabilization (Gilby and McKellar, 1976)

In contrast to the inorganic elements, carbon, hydrogen, nitrogen and sulfur were much more abundant in house fly exuviae than in face fly exuviae (Table 2) These results indicated that organic components such as proteins and catechols constitute a greater percentage of the weight of sclerotized cuticle relative to mineralized cuticle

Amino acid and glucosamine compositions

The amino acid and glucosamine compositions of acid hydrolyzed puparial cuticle of the three Dipteran species are shown in Table 3 There was no significant difference in glucosamine (chitin) content between the three species Large differences existed between the amino acid compositions of mineralized and sclerotized puparia Face fly cuticle contained two to three fold less total amino acids (or protein) than did sclerotized house fly and stable fly cuticles Bodnaryk (1972) reported similar differences between *M autumnalis* and *M domestica* in amino acid composition The trend in overall amino acid (protein) content (stable fly > house fly > face fly) was opposite to that of mineral content which indicated that inorganic salts substitute for protein in cuticle The only amino acid present at a comparable level in the exuviae of all three species was glycine The major incongruity found between the various flies was the absence of β-alanine in face fly cuticle, as previously shown by Bodnaryk (1972) β-Alanine is also absent from the calcified puparia of *M fergusoni* (Gilby and McKellar, 1976) β-Alanine is important in cuticular tanning due to its conjugation with dopamine as NBAD (Hopkins *et al*, 1982, 1984, Kramer *et al*, 1984) The β-alanine results are apparently related to the presence or absence of NBAD in puparial cuticle of the three species (see next section)

Catechol composition

When compared to *M domestica* and *S calcitrans*, the free catechol content of *M autumnalis* puparial exuviae was strikingly dissimilar (Table 4) Dopamine was the major catechol in mineralized cuticle whereas much larger quantities of dopamine and other catechols (> 70–170 fold higher) were found in the

Table 2 Major organic elements as percentage of dry weight of puparial exuviae of face fly and house fly*

Element	Face fly	House fly	Ratio (HF FF)
Carbon	9 73 ± 0 04	45 33 ± 0 06	4 6
Hydrogen	3 31 ± 0 01	6 75 ± 0 05	2 0
Oxygen	23 72 ± 0 07	29 70 ± 0 03	1 3
Nitrogen	1 52 ± 0 08	9 50 ± 0 01	6 3
Sulfur	< 0 1	0 24 ± 0 02	> 2 4

*Mean of three determinations ± SE

Table 3 Amino acid and glucosamine compositions (μmol g) of puparial exuviae from three species of Diptera*

Amino acid	Face fly	House fly	Stable fly
Aspartic acid amide	72 8 ± 2 9	208 4 ± 26 7	284 7 ± 15 9
Threonine	41 3 ± 1 5	88 6 ± 10 1	117 2 ± 6 9
Serine	36 6 ± 0 2	78 5 ± 8 0	124 3 ± 10 6
Glutamic acid amide	86 3 ± 3 1	214 5 ± 25 0	259 2 ± 14 9
Proline	46 3 ± 0 9	115 7 ± 12 2	137 9 ± 8 4
Glycine	303 8 ± 9 9	319 5 ± 57 8	340 8 ± 10 6
α-Alanine	57 2 ± 0 8	123 4 ± 16 7	194 1 ± 5 4
Valine	53 8 ± 1 9	134 4 ± 12 2	170 9 ± 7 7
Methionine	4 6 ± 0 4	9 1 ± 1 7	12 8 ± 1 7
Isoleucine	25 2 ± 1 2	58 3 ± 7 5	89 0 ± 2 0
Leucine	28 7 ± 1 6	76 1 ± 10 2	102 8 ± 1 3
Tyrosine	22 9 ± 0 8	53 6 ± 8 7	64 7 ± 1 5
Phenylalanine	17 8 ± 1 2	47 8 ± 7 9	66 7 ± 1 9
β-Alanine	< 5	299 2 ± 43 1	327 6 ± 41 0
Histidine	41 7 ± 4 7	227 4 ± 37 6	301 4 ± 22 5
Lysine	27 5 ± 3 3	85 8 ± 16 3	101 0 ± 3 8
Arginine	17 1 ± 2 9	41 2 ± 16 1	42 3 ± 3 5
Total amino acid relative ratio	0 32	0 79	1 0
Glucosamine	43 3 ± 4 7	36 6 ± 14 7	57 2 ± 22 9

*Mean values ± SE from three analyses of puparia hydrolyzed for 20 hr *in vacuo* in 6 M HCl containing 1% phenol Tryptophan and cysteine were not determined

Table 4 Catechol composition (nmol/g) of puparial exuviae from three species of Diptera*

Catechol	Face fly	House fly	Stable fly	Detection limits
NBAD	< 1 2	312 0 ± 56 7	774 0 ± 126 8	1 2
Dopamine	5 0 ± 0 8	21 4 ± 1 5	46 4 ± 5 5	1 9
DOPA	< 0 7	11 4 ± 0 5	28 6 ± 24 4	0 7
NADA	< 0 9	27 2 ± 10 9	21 8 ± 5 0	0 9
DOPAC	< 0 5	4 6 ± 1 8	16 7 ± 3 1	0 5
Total	5 0	376 6	887 5	

*Mean value ± SE for 5–6 determinations except for house fly DOPA value where three determinations were made NBAD *N*-β-alanyldopamine dopamine 3 4-dihydroxyphenethylamine, DOPA 3 4-dihydroxyphenylalanine NADA *N*-acetyldopamine DOPAC 3 4-dihydroxyphenylacetic acid

sclerotized cuticles Stable fly cuticle had the highest total concentration of catechols being more than two times higher than house fly and nearly 200 times higher than face fly *N*-β-Alanyldopamine (NBAD) was the major catechol in puparial exuviae of the stable fly and house fly but it was undetected in the face fly This result is in accord with the presence or absence of β-alanine in the exuviae of these species (Table 3) There were apparently no ring hydroxyl conjugated catechols in Dipteran cuticle because no increase in catechol level occurred after mild acid hydrolysis (1 M HCl at 100 °C for 10 min) *N*-Acetyldopamine (NADA), the major catechol precursor for schlerotization of puparial cuticle of *Calliphora* and *Drosophila* (Karlson and Sekeris, 1962, Sekeris and Herrlich 1966), appears to be of only minor importance as a free constituent in cuticle of *Musca* and *Stomoxys* since it is 10 to 40 times less abundant than NBAD

Dopamine levels during pupariation

If catechols are important for stabilization of the face fly puparium, one would expect their levels to be highest at the time of pupariation when the old larval cuticle is being transformed into the puparial case In the tobacco hornworm, *Manduca sexta* (L) (Hopkins *et al*, 1982, 1984) and the red flour beetle, *Tribolium castaneum* (Herbst) (Kramer *et al* 1984), cate-

Table 5 Dopamine and *N*-β-alanyldopamine levels (nmol g wet tissue) in face fly and house fly cuticles during pupariation*

	Dopamine		NBAD	
Stage of development	Face fly	House fly	Face fly	House fly
Wandering larvae	35 3 ± 4 2	1 0 ± 0 4	< 1 2	2 5 ± 0 6
Puparia				
0–5 hr	232 5 ± 14 8	15 6 ± 5 3	< 1 2	265 3 ± 153 0
36 hr	11 1 ± 1 3			
48 hr	3 4 ± 0 6			
Post eclosion	5 0 ± 0 8	21 4 ± 1 4	< 1 2	312 0 ± 56 7

*Mean values ± SE for 3–6 determinations Six cuticles pooled for each determination NBAD = *N* β-alanyldopamine

Table 6 Percentage distribution of radioactivity recovered from adults and puparial exuviae from [2-^{14}C]DOPA injected larvae of face fly and house fly*

Species	Adult	Puparial exuviae	
		Soluble	Insoluble
Face fly	78 5 ± 13 3	3 0 ± 1 0	18 4
House fly	42 6 ± 19 3	4 4 ± 0 7	52 9 ± 14 5

*Larvae in wandering stage injected with 23,500 cpm (face fly) and 1400 cpm (house fly) Total recovery of counts after adult eclosion ~85% Mean values ± SEM for 2–3 determinations except for face fly puparial exuviae insoluble tissue for which a single determination of pooled samples was performed

cholamines such as dopamine, NADA and NBAD increase shortly before cuticle stabilization and subsequently decline

We measured the catechol content of face fly and house fly cuticles during pupariation (Table 5) Cuticle from feeding face fly larvae did not contain appreciable amounts of catechols (< 1 nmol/g) The only catechols that increased to significant levels thereafter were dopamine and to a lesser extent norepinephrine (data not shown) About the time of pupariation dopamine increased six-fold over the level in cuticle from wandering larvae The house fly accumulated NBAD into its puparial cuticle at a comparable level to dopamine in the face fly As the face fly puparial cuticle stabilized over a 48 hr period, the dopamine titre fell to approx 2% of its highest level In contrast, NBAD remained elevated in house fly cuticle after stabilization It appears that catechols may act as filling material in house fly cuticle while inorganic salts do the same in face fly puparia These results indicate that the face fly and house fly use dopamine and NBAD, respectively, to sclerotize puparial cuticle

Incorporation of catecholamines into fly puparia

Although dopamine is extractable from face fly puparial exuviae, such observation does not provide any indication of how much catechol becomes covalently incorporated into stabilized cuticle as should occur during sclerotization (Brunet, 1980) To determine the amount of unextractable catechol that is presumably covalently linked to the cuticular chitin-protein matrix, we injected [2-^{14}C]DOPA into wandering larvae of both *M autumnalis* and *M domestica* and determined the distribution of radioactivity after adult eclosion had taken place Presumably DOPA is decarboxylated to dopamine and incorporated into various tissues Over 20% of the radioactivity was recovered in the puparial exuviae of the face fly and the remainder was present in the adult fly (Table 6) More than 80% of the exuvial radiocarbon was insoluble (unextractable in 1 2 M HCl) while only a small percentage remained as free catechols or other soluble metabolites As was expected from a species that relies more heavily on sclerotization for cuticle stabilization, the house fly exhibited a more than two-fold greater percentage of radiocarbon in the insoluble fraction from the puparia than the face fly It appears that catechols become covalently incorporated into the stabilized cuticle and that sclerotization is indeed an integral process of both house fly and face fly pupariation, but to much different degrees

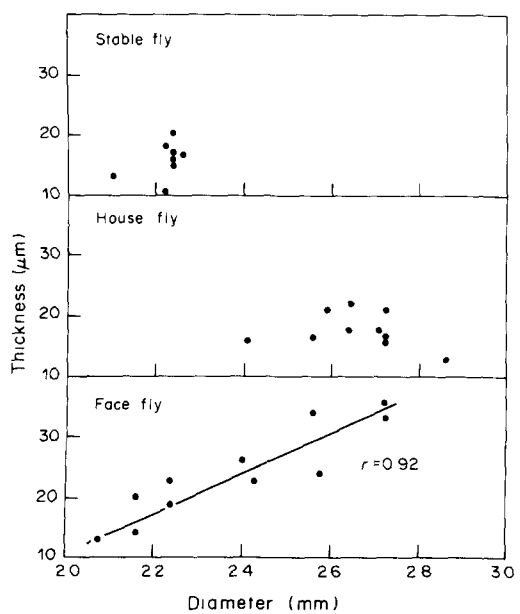

Fig 1 Relationship between thickness (μm) and diameter (mm) for puparial exuviae of stable fly, house fly and face fly

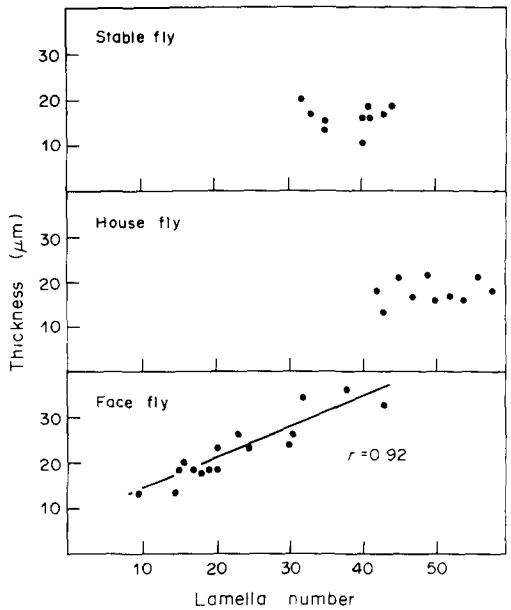

Fig 2 Relationship between thickness (μm) and number of lamellae for cuticle of stable fly, house fly and face fly

Thickness, hardness and density of puparial exuviae

Thickness of face fly puparial cuticle increased significantly as puparial diameter increased (Fig 1, $r = 0\ 92$) No significant relationship between thickness and diameter was observed for either house fly or stable fly puparia The thickness of house fly and stable fly cuticles varied from only 11 to 22 μm, while that of face fly ranged from 13 to 36 μm The number of lamellae in face fly puparial cuticle ranged from 10 to 43 and increased significantly as thickness increased (Fig 2, $r = 0\ 92$) The number of lamellae

Table 7 Relative hardness of puparial exuviae from face fly house fly and stable fly*

Species	Breaking force (g)	Ratio of breaking force to thickness (g μm)
Face fly	17 54 ± 2 23	0 61 ± 0 05
House fly	38 54 ± 0 92	2 08 ± 0 13
Stable fly	28 29 ± 0 86	1 82 ± 0 14

*Mean values ± SEM n = 28–30 and 10 for breaking force and ratio determinations, respectively

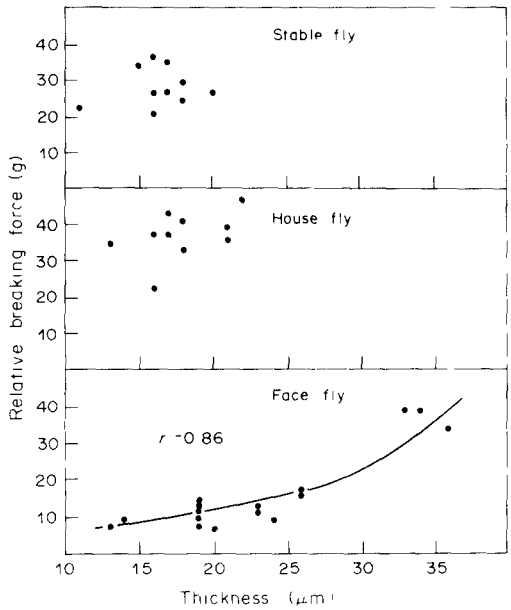

Fig 3 Relationship between relative breaking force (g) and thickness (μm) for cuticle of stable fly house fly and face fly

were higher in house fly (42–50) and stable fly (32–44) cuticles However, no relationship between lamellae and thickness was observed in these species The hardness of mineralized puparial exuviae was significantly different from that of sclerotized exuviae The average force required to fracture face fly cuticle was approximately two-fold less than those of house fly or stable fly (Table 7) When the breaking forces were normalized to the same cuticular thickness, the differences were even more pronounced Sclerotized cuticle was at least three times harder than mineralized cuticle The breaking force of face fly cuticle equaled or exceeded those of house fly and stable fly only when the thickness of mineralized cuticle was about 1 5 times greater than sclerotized cuticle (Fig 3) A highly significant correlation (r = 0 86) was observed between the breaking force of face fly cuticle and its thickness In contrast, there was no correlation between these parameters for house fly or stable fly cuticles

The densities of face fly and house fly puparial exuviae were calculated by dividing their weight by their puparial shell volume Face fly exuviae exhibited a more than 20% higher mean density than house fly exuviae (2 64 ± 0 16 mg mm^{-3} vs 2 17 ± 0 25 mg mm^{-3}, respectively, P = 0 0038)

DISCUSSION

Although the proposal that mineralization supplants sclerotization in face fly puparia (Fraenkel and Hsiao, 1967) has been generally accepted, it has been suggested that this conclusion is not entirely warranted (Darlington *et al*, 1983) That a completely different mechanism has supplanted a common insect pathway used in the formation of such an important structure as cuticle seemed surprising Therefore we addressed the question of whether, in addition to mineralization, sclerotization of face fly puparia also takes place It was established that (1) tyrosine metabolites (catecholamines) accumulate in this cuticle at the time of pupariation and diminish thereafter and (2) the metabolites become tightly bound (probably covalently incorporated) as the cuticle stabilizes Thus we conclude that sclerotization does occur in *M autumnalis* puparia

The observation that dopamine is a predominant catecholamine in an insect cuticle is a novel one An acylated derivative of dopamine such as NADA or NBAD is most frequently observed in high titre in other species such as house fly tobacco hornworm (Hopkins *et al*, 1982 1984) and red flour beetle (Kramer *et al*, 1984) although an oxidative degradation product of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), may be detected at high levels In adult cuticle of the black mutant of the red flour beetle, dopamine is a major catechol (Kramer *et al*, 1984) Its presence in the cuticle gives rise to an overproduction of melanin which results in the black colouration The absence of an appreciable colour in the face fly puparium indicates that dopamine is used for the production of protein crosslinking agents instead of pigments (Aso *et al*, 1984) NBAD, on the other hand is the predominant precursor for a sclerotizing agent in house fly and stable fly puparia while NADA has a similar role in *Calliphora* and *Drosophila* (Karlson and Sekeris 1962 Sekeris and Herrlich, 1966)

The quantitative differences in the amounts of catechols, amino acids and inorganic salts in fly cuticle reflects the relative importance of mineralization and sclerotization in puparial stabilization Face fly puparia have much higher levels of minerals and much lower catechols and amino acids in a cuticle that stabilizes and hardens primarily by mineral salt deposition The house fly and stable fly, which employ a sclerotization mechanism to stiffen and harden the puparial wall have much higher concentrations of amino acids and catecholamines and correspondingly lower mineral levels Our data indicate that the three Dipteran species utilize both mechanisms of stabilization but to very different extents

Stabilized house fly and stable fly cuticles are impregnated with 75 and nearly 200 times more extractable catechols, respectively than face fly cuticle Radiolabeling experiments using L-DOPA as a precursor show that the extent of covalent incorporation of catechols into face fly and house fly cuticles is 6 to 12 times higher than free catechol concentrations Assuming that L-DOPA is the primary precursor for dopamine synthesis, the house fly could incorporate as much as 4500 nmol catechol/g of

cuticle while the face fly would incorporate only about 30 nmol/g Structural proteins and chitin may be crosslinked by the catecholamine-derived quinones to form a matrix into which other stabilizing factors or fillers may be added The face fly deposits more minerals while the house fly and stable fly infuse more catecholamines and proteins into their cuticles Minerals, catecholamines and proteins are apparently filling materials that are used to give cuticle the required chemical and physical properties

Sclerotization involves several enzymes including tyrosinase, DOPA decarboxylase and acyl transferases which transform tyrosine into catecholamines and quinones Quinonoid metabolites are probably the electrophilic crosslinking agents of cuticular structural proteins (Brunet, 1980, Hopkins *et al*, 1984, Aso *et al*, 1984) In the case of *M autumnalis*, injection of radiolabeled DOPA into larvae leads to its incorporation into the puparial cuticle Since dopamine and not DOPA is present in puparial cuticle, DOPA is most likely converted to dopamine by DOPA decarboxylase in the face fly DOPA decarboxylase activity increases to high levels during pupariation of Diptera (Shaaya and Sekeris, 1965, Lunan and Mitchell, 1969, Marsh and Wright, 1980) Puparial cuticular proteins of a flesh fly, *Sarcophaga bullata*, acquire radiolabel when larvel instars are administered [7-^{14}C]dopamine (Lipke *et al*, 1981) Tyrosinase may also produce quinones from DOPA or dopamine that would be incorporated into the insoluble fraction of cuticle We regard the oxidation and direct uptake of non-oxidized DOPA as less plausible than decarboxylation to dopamine given the preponderance of dopamine in face fly cuticle and NBAD in house fly and stable fly cuticles Another possible fate of DOPA is incorporation into the cuticle as melanin This seems unlikely since the puparia of house flies and stable flies are devoid of black pigments

The failure of free tyrosine titres to diminish during face fly development was interpreted to mean that sclerotization did not occur since a large decrease was observed in the house fly (Fraenkel and Hsiao, 1967) However, tyrosine conjugates or other derivatives may have been excluded from that analysis Conjugates that are more soluble than tyrosine and that protect the phenolic moiety from hydroxylation are common storage forms prior to moulting (Brunet, 1980, Kramer *et al*, 1980, Lu *et al*, 1982) A determination of tyrosine metabolites and their fate has shown that *M autumnalis* does undertake a process of stabilization based upon use of covalent bonding of catecholic derivatives into puparial cuticle Unclear however is the relative importance of sclerotization since Fraenkel and Hsiao (1967) have reported that only a soft, transparent and colourless cuticle remains after removal of the mineral components with acid Darlington *et al* (1983) also report that puparia become soft and semi-transparent after treatment with 0 1 N HCl

The stabilization of crab cuticle by mineralization may be a homologous process to the mineralization of insect cuticle Catechols typically found in tanning insect tissues, including NADA and *N*-acetylnorepinephrine, are found in haemolymph and cuticle of crustaceans (Vacca and Fingerman, 1975) The occurrence of both mineralization and sclerotization in those arthropods is well documented Dendinger and Alterman (1983) showed that the initial postmoult tensile strength is due to sclerotization and that subsequent investment of the cuticle with salts causes brittleness to increase Hepburn *et al* (1975) suggest that mineralization of cuticle imparts greater resistance to compressability

Even though face fly exuviae contain 17-fold more ash than house fly exuviae, their density is only moderately greater (20%) Apparently sclerotized cuticle is more densely packed with organic material than mineralized cuticle with inorganic material When compared in terms of density, thickness and hardness, cuticle stabilized primarily with protein and catechols is both lighter and stronger than cuticle stabilized primarily with minerals The differences in physical properties no doubt played a major role in the preponderant selection of sclerotization instead of mineralization as a cuticular stabilization mechanism for insects The face fly appears to compensate for the use of a more brittle material (minerals) by adding more of that material to the cuticle Face fly puparia of larger diameter are thicker due to an increased number of lamellae This process attains a cuticular hardness similar to that of house fly and stable fly

Additional research may show that specific mechanical properties (for example, hardness, brittleness, tensile strength) of a complex composite material such as cuticle may be ascribed to individual components such as scleroproteins, chitin, catechols, minerals and lipids When calcium was extracted from crab cuticle, tensile strength increased by nearly 50% (Dendinger and Alterman, 1983) Although the puparial breaking force data are not directly comparable to tensile strength data, both types of data suggest that minerals harden cuticle less effectively than proteins and catechols When biochemical data are correlated to physical and ultrastructural properties of cuticle, the roles of specific cuticular biochemicals will be revealed in greater detail

Acknowledgements—We thank Thomas D Morgan and John Krchma Department of Entomology, Kansas State University, and Dana Wright and Robert Rousser, U S Grain Marketing Research Laboratory, for technical assistance, Dr R Carl Hoseney and Wayne R Moore, Department of Grain Science and Industry, Kansas State University for assistance with the measurements of cuticular hardness, and Dr Lynn Riddiford, University of Washington, and Dr Peter Dunn, Purdue University, for critical comments

Contribution no 84-476-J from the Departments of Entomology and Biochemistry, Kansas Agricultural Experiment Station, Manhattan, KS 66502 Cooperative investigation between Agricultural Research Service, U S Department of Agriculture and the Kansas Agricultural Experiment Station Mention of a proprietary product in this paper does not imply its approval by the USDA to the exclusion of other products that may also be suitable Supported in part by research grant PCM 8003859-AO2 from the National Science Foundation, 82-1-1055 from the USDA Competitive Research Grant Program and 82-CRSR-2-2017 from the USDA Cooperative State Research Grant Program

REFERENCES

Aso Y, Kramer K J, Hopkins T L and Whetzel S Z (1984) Properties of tyrosinase and DOPA quinone imine

conversion factor from pharate pupal cuticle of *Manduca sexta* (L.). *Insect Biochem.* **14**, 463–472.

Bodnaryk R. P. (1972) Amino-acid composition of the calcified puparium of *Musca autumnalis* and sclerotized puparium of *Musca domestica*. *Insect Biochem.* **2**, 119–122.

Brunet P. C. J. (1980) The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. *Insect Biochem.* **10**, 467–500.

Darlington M. V., Meyer H. J., Graf G. and Freeman T. P. (1983) The calcified puparium of the face fly *Musca autumnalis* (Diptera: Muscidae). *J. Insect Physiol.* **29**, 157–162.

Dendinger J. E. and Alterman A. (1983) Mechanical properties in relation to chemical constituents of post molt cuticle of the blue crab *Callinectes sapidus*. *Comp. Biochem. Physiol.* **75A**, 421–424.

Dennell R. (1958) The amino acid metabolism of a developing insect cuticle: the larval cuticle and puparium of *Calliphora erythrocephala*. I. Changes in amino acid composition during development. *Proc. R. Soc.* **148**, 270–279.

Fraenkel G. and Hsiao C. (1967) Calcification, tanning and the role of ecdysone in the formation of the puparium of the face fly *Musca autumnalis*. *J. Insect Physiol.* **13**, 1387–1394.

Fraenkel G. and Rudall K. M. (1940) A study of the physical and chemical properties of the insect cuticle. *Proc. R. Soc.* **129**, 1–35.

Fukushi Y. and Seki T. (1965) Differences in amino acid compositions of pupal sheaths between wild and black pupa strains in some species of insects. *Jap. J. Genet.* **40**, 203–208.

Gilby A. R. and McKellar J. W. (1976) The calcified puparium of a fly. *J. Insect Physiol.* **22**, 1465–1468.

Hackman R. H. and Goldberg M. (1971) Studies on the hardening and darkening of insect cuticles. *J. Insect Physiol.* **17**, 335–347.

Hepburn H. R., Joffe I., Green N. and Nelson K. J. (1975) Mechanical properties of crab shell. *Comp. Biochem. Physiol.* **50A**, 551–554.

Hopkins T. L., Morgan T. D., Aso Y. and Kramer K. J. (1982) *N*-β-Alanyldopamine: major role in insect cuticular tanning. *Science* **217**, 364–366.

Hopkins T. L., Morgan T. D. and Kramer K. J. (1984) Catecholamines in hemolymph and cuticle during larval, pupal and adult development of *Manduca sexta* (L.). *Insect Biochem.* **14**, 533–540.

Karlson P. and Sekeris C. E. (1962) *N*-Acetyldopamine as sclerotizing agent of the insect cuticle. *Nature* **195**, 183–184.

Kramer K. J., Hopkins T. L., Ahmed R. F., Mueller D. and Lookhart G. (1980) Tyrosine metabolism for cuticle tanning in the tobacco hornworm *Manduca sexta* (L.) and other Lepidoptera: Identification of β-D-glucopyranosyl-*O*-L-tyrosine and other metabolites. *Archs. Biochem. Biophys.* **205**, 146–155.

Kramer K. J., Morgan T. D., Hopkins T. L., Roseland C. R., Aso Y., Beeman R. W. and Lookhart G. L. (1984) Catecholamines and β-alanine in the red flour beetle *Tribolium castaneum*: Roles in cuticle sclerotization and melanization. *Insect Biochem.* **14**, 293–298.

Lipke H., Strout K., Henzel W. and Sugumaran M. (1981) Structural proteins of Sarcophagid larval exoskeleton: Composition and distribution of radioactivity derived from 7-^{14}C-dopamine. *J. biol. Chem.* **256**, 4241–4246.

Lipke J., Sugumaran M. and Henzel W. (1983) Mechanisms of sclerotization in dipterans. *Adv. Insect Physiol.* **17**, 1–85.

Lu P., Kramer K. J., Seib P. A., Mueller D. D., Ahmed R. and Hopkins T. L. (1982) β-D-Glucopyronosyl-*O*-L-tyrosine: synthesis, properties and titre during insect development. *Insect Biochem.* **12**, 377–381.

Lunan K. D. and Mitchell H. K. (1969) The metabolism of tyrosine-*O*-phosphate in *Drosophila*. *Archs. Biochem. Biophys.* **132**, 450–456.

Marsh J. L. and Wright T. R. F. (1980) Developmental relationship between dopa decarboxylase, dopamine acetyltransferase and ecdysone in *Drosophila*. *Dev. Biol.* **80**, 379–387.

Neville A. C. (1975) Biology of the arthropod cuticle. In *Zoophysiology and Ecology*, Vol. 4. 5. Springer-Verlag, New York.

Roseland C. R., Beeman R. W., Kramer K. J. and Hopkins T. L. (1983) Microinjection of amino acids in *Tribolium*. *Tribolium Inform. Bull.* **23**, 132.

Sekeris C. E. and Herrlich P. (1966) Zum Tyrosinstoffwechsel der Insekten XIII. Der Tyrosinstoffwechsel von *Tenebrio molitor* und *Drosophila melanogaster*. *Hoppe-Seyler's Z. physiol. Chem.* **344**, 267–275.

Shaaya E. and Sekeris C. E. (1965) Ecdysone during insect development III. Activities of some enzymes of tyrosine metabolism in comparison with ecdysone titer during development of the blowfly *Calliphora erythrocephala* Meig. *Gen. comp. Endocr.* **5**, 35–39.

Vacca L. L. and Fingerman M. (1975) The mechanism of tanning in the fiddler crab *Uca pugilator*. I. Tanning agents and protein carriers in the blood during ecdysis. *Comp. Biochem. Physiol.* **51B**, 475–481.

Vincent J. F. V. and Hillerton H. F. (1979) The tanning of insect cuticle: A critical review and a revised mechanism. *J. Insect Physiol.* **25**, 653–658.